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Full Length Article

Chemical variation of leaf essential oil at different stages of plant growth and *in vitro* antibacterial activity of *Thymus vulgaris* Lamiaceae, from Iran

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ABSTRACT

The essential oil components of the leaves were isolated by hydrodistillation from *Thymus vulgaris* (T.) Lamiaceae, at different stages of plant growth. The essential oils from *T.* Lamiaceae leaves were obtained in yields of 0.83–1.39% (w/w). The oils were studied by gas chromatography mass spectrometry (GC/MS) and thirty-six components were identified in the oil. The major components in the leaf oils were: thymol (38.23–63.01%), o-cymene (5.56–15.47%), γ-terpinene (4.43–7.17%), borneol (1.72–6.65%), 4-terpineol (1.24–5.16%) and 1,8-cineole (0.09–1.54%). The results showed that the oil yield and the major constituents' percentage of the leaf were different at different stages of plant growth. The essential oils of *T.* Lamiaceae leaves were tested against five strains of Gram positive bacteria (g^+) and five strains of Gram negative bacteria (g^-). The average minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of essential oils were determined using agar dilution method against the organisms by agar dilution method.
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1. Introduction

Even today, the number of plants that have been extensively studied is relatively very few and the vast majority has not been studied at all. Investigations of aromatic and medicinal plants enable finding plants producing effective essential oils that have already found a considerable range of applications (Mohammed and Al-Bayati, 2009). Traditional medicine is widespread globally and it is the almost exclusive source of primary health care for 65% of the world's population (Nezhadali et al., 2010a,b,c). Herbal remedies have long-standing roles in

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treating disease, and in developing countries are widely taken by and administered to women by traditional birth attendants during pregnancy. In the last decade, ethno pharmacological studies showed that herbal remedies have been employed routinely during pregnancy and childbirth (Johns et al., 1990).

The essential oils possess antibacterial, antifungal, antiviral, antioxidant and wide spectrum of pharmacological activities. These properties of essential oils are used in pharmacy and food industry. The essential oils became official drugs in many countries and have been documented in their pharmacopoeias. The essential oils have found the widest use in the treatment of infectious pathologies of the respiratory and gastrointestinal systems, urinary tract as well as at various skin diseases. Various species of the *Thymus* genus were reported to be strongly antibacterial, antifungal and antioxidant activities. These properties depend on the essential oil composition. Thyme oils are listed in pharmacopoeias of Europe, Germany and United Kingdom and used as natural preservatives in the food industry. The volatile components are important in determining the biological activity of *Thymus* species. *T.* is a perennial herb of Lamiaceae family. It has dark green leaves with light mauve-pink flowers in early Summer (Nezhadali et al., 2010a,b,c). Thymol, , which is the principal constituent of thyme oil have been reported to act as antioxidant, antimicrobial agent, antifungal agent treatment for respiratory tract diseases, wound healing, a stomachic carminative, diuretic, urinary disinfectant and vermifuge. The composition and quantity of essential oil from a particular species of thyme plant could be markedly affected by harvesting season, geographical environment and other agronomical factors. The beneficial effects of thyme are well known from ancient times and consumption of its extract is recommended all over the world. It is considered as the main ingredient of many phytopreperations and commonly used as water extracts for its pharmacological activities and thus, have a very important role in phytotherapy. Recently, Thyme has become one of the most important medicinal plants used as a natural additive in poultry and livestock feeding studies. Such studies showed that thyme plant could be considered as an alternative natural growth promoter for poultry instead of antibiotics (Abu-darwish and Abu-dieyeh 2009). The market demand for thyme is rather high, yearly estimates running at about 500 tones in USA and 1000 tones in Europe. Owing to a general popularity of the use of natural substances instead of synthetic compounds, an increase in that demand is predictable. The yield of plant material, the essential oil content and quantitative composition of *Thymus vulgaris* can be influenced by harvest time, ecological and climatically conditions. It has been reported that a fairly tight correlation exists between the soil type and the chemotypic structure of the thyme population growing on it. Where the soil type varies, distinct differences among chemotypes can be found over a few meters. Since the altitude can also be considered as a major factor influencing the physiological and chemical responses of plants, a correlation is attempted between the altitude where aromatic plants occur and their yield in essential oils. However, relatively high values of yield occur in a wide range of altitudes, from 600 to 1900 m (in Greece). Concerning the other chorological groups, it was found that the range of essential oil yield is fairly limited. For Balkan and Eurasiatic elements, in particular, yield does not seem to be related to altitude as in the case of the Mediterranean elements. Knowledge of the factors such as genetic or environmental that influence the essential oil content is insufficient, and the same holds for the role they exerted and now exert in the ecological complex. Region and altitude seems to play a role in the case of oil rich and oil-intermediate aromatic plants, affecting their essential oil content. It does not seem to influence oil poor plants. Drug yield, essential oil content and composition in *T.* plants showed big variation from years to years because of perennial plants.

In this study we investigated chemical variation of the leaves essential oil at different stages of plant growth and *in vitro* antibacterial activity of *T.* Lamiaceae.

2. Material and methods

2.1. GC/MS conditions

The essential oil composition was analyzed using a Shimadzu QP5050 GC/MS with DB-5 capillary column. The analysis program and conditions were as follows: helium at 1.7 mL/min as a carrier gas; the injection volume was 0.1 μL; ionization potential, 70 ev; the initial temperature of the column was kept at 60 °C (an usual temperature in the analysis of herbal plants) for 1 min and programmed to 140 °C at a rate of 3 °C/min and kept constant at 250 °C (column cleaning step) for 3 min.

2.2. Quantification and identification

N-alkenes mixture was performed under the GC/MS temperature condition program to calculate the Kovat's indexes (RI). These observations were further supported by mass spectral data, literature, and NIST computer library which confirmed the identity of each component. The relative percentage of the oil constituents was calculated.

2.3. Essential oil extraction

The plant sample after collected was transferred to the herbarium of Botanical Research Center of Payame Noor University, Mashhad, Iran. The plant was identified using valid references such as botanical Flora Iranica (Rechinger, 1998) and Flora of Iran (Mozaffaryan, 1999). The leaves of *T. vulgaris* Lamiaceae, at different stages of plant growth were collected from Barzo Mountain Shirvan (North Khorasan Province of Iran) at altitude of 1700 m in June–July 2010 (four samples). The collected samples on 6th-June-2010, 13th-June-2010, 3rd-July-2010, and 16th-July-2010 were named S_1, S_2, S_3, and S_4 respectively. The plant leaves were dried at room temperature in a shadow place for 6 days. The leaves of each stage (45 g) were hydrodistillated in a Clevenger-type apparatus for 4 h according to the method recommended in the British Pharmacopoeia. The extracted oil was deoxygenated under nitrogen gas and stored in a sealed vial at low temperature until the analysis time.

2.4. Antimicrobial activity

2.4.1. Disk diffusion assay

The used bacterial strains, which were in this study, were obtained from the Persian Type Culture Collection (PTCC). The

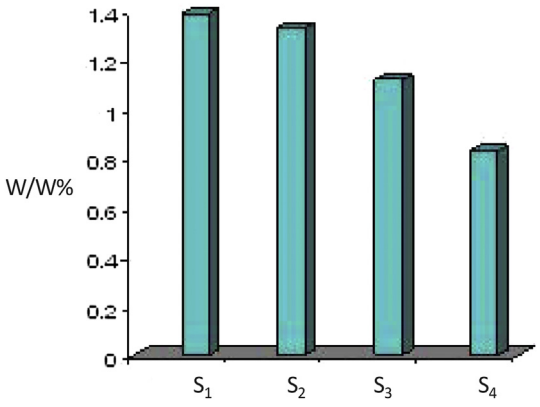

Fig. 1 – The percentage yield of essential oil obtained by hydrodistillation from *Thymus vulgaris* Lamiaceae leaves, at different stages of plant growth.

essential oils of the *T.* Lamiaceae leaves, were tested against five strains of Gram positive bacteria and five strains of Gram negative bacteria. These bacteria were as: *Staphylococcus aureus* (PTCC 1113,g^+), *Staphylococcus epidermidis* (PTCC 1435,g^+), *Streptococcus pyogenes* (PTCC 1447,g^+), *Enterococcus faecalis* (PTCC 1394,g^+), *Listeria monocytogenes* (PTCC 1298,g^+), *Escherichia coli* (PTCC 1330,g^-), *Klebsiella pneumonia* (PTCC 1053,g^-), *Proteus vulgaris* (PTCC 1079,g^-), *Yersinia enterocolitica* (PTCC 1477,g^-) and *Pseudomonas aeruginosa* (PTCC 1074,g^-).

The minimal inhibitory concentration (MIC) for each sample was determined by disk diffusion method (Ncube et al., 2008) at a concentration testing range of $5 \times 10–5 \times 10^5$ μg ml^{-1} (Nezhadali et al., 2010a,b,c). The standard bacterial strains were primarily incubated in a nutrient broth at a temperature of 37 °C until reaching 0.5 on the McFarland scale. Then the bacteria were transferred to Muller–Hinton media following the Kirby–Bauer method (Jones et al., 2001). In each plate, disks with the different dilution of the essential oils were placed. The essential oils diluted with dimethyl sulfoxide then the disks smeared with the solution. The blank disk including DMSO without essential oil was used as a negative control while gentamycine and kanamycin were used as positive controls (Nezhadali et al., 2009). All plates were then incubated at 37 °C for 24 h, after which the bacterial inhibitory halos, if present, were measured in millimeters.

2.4.2. *Broth dilution assay*

In order to evaluate if the bacterial inhibitory halos were the result of bacteriostatics or bacteriocidal effects, the MBC assay was done. In this step, broth dilution method was used for MBC assay. The MBC was the lowest concentration of the antimicrobial agent that showing 99.9% bacterial growth inhibition. For this purpose, 200 μl of each sample in 1.5 ml micro tube was incubated at 37 °C for 24 h until solvent evaporated. Then, 200 μl of each bacterium sample and 400 μl nutrient broth culture medium were added to micro tubes, and were incubated at 37 °C for 24 h. After incubation time, the turbidity of each micro tube was considered and in order to determine MBC, an aliquot (0.1 ml) from the micro tubes that showed growth inhibition was placed on an agar plate that does not contain any antimicrobial agent. The experiments were repeated three times and the average value of MIC and MBC were reported.

Table 1 – The chemical compositions of essential oil of *T.* Lamiaceae leaves at different stages of plant growth.

No.	Compound	KI[a]	Percentage			
			S_1	S_2	S_3	S_4
1	α-Thujene	951	0.34	0.48	0.24	0.05
2	α-Pinene	957	0.56	0.63	0.44	0.59
3	Camphene	970	0.87	0.70	0.86	0.96
4	β-Pinene	992	0.25	0.23	0.25	0.29
5	β-Myrcene	1006	0.97	1.07	1.63	0.85
6	α-Phellandrene	1017	0.12	0.27	0.38	2.09
7	α-Terpinen	1032	1.09	0.90	0.36	0.23
8	O-cymene	1053	5.56	9.88	13.24	15.47
9	1,8-cineole	1060	1.54	1.16	0.09	0.10
10	γ-Terpinene	1089	4.43	7.17	6.92	5.48
11	*cis*-β-Terpineol	1089	1.07	1.42	1.33	0.34
12	Terpinolene	1103	0.36	0.24	0.47	0.60
13	Linalool	1117	0.49	0.46	0.75	0.60
14	Isopulegol	1135	0.08	0.09	0.34	0.43
15	Camphor	1162	2.91	0.26	2.09	2.87
16	Borneol	1187	6.65	1.72	6.63	3.84
17	4-Terpineol	1193	5.16	1.24	2.89	4.01
18	Isoborneol	1198	2.03	1.07	4.24	4.09
19	Thymol methyl ether	1254	0.98	0.36	1.57	1.54
20	Verbenone	1268	0.18	0.13	0.28	0.20
21	α-Terpineol	1279	0.33	0.06	0.27	0.19
22	Dihydro carvone	1293	0.55	0.04	0.29	0.11
23	Thymol	1380	52.77	63.01	45.4	38.23
24	Eugenoi	1401	0.44	0.15	0.47	0.35
25	β-Caryophyllene	1468	0.17	0.14	0.20	0.38
26	α-Beragmotene	1460	0.51	0.04	0.09	0.06
27	Germacrened	1509	0.89	0.30	1.12	0.76
28	γ-Elemene	1515	0.71	0.11	0.28	0.24
29	β-Bisabolene	1537	0.92	0.57	0.96	0.78
30	Delta-cadinene	1551	0.91	0.26	0.44	0.40
31	Caryophyllene oxide	1588	0.16	0.04	0.26	1.67
32	Spathulenol	1608	0.72	0.46	0.72	0.88
33	Aromadendrene oxide	1622	0.04	0.03	0.08	0.08
34	Cadinol	1646	0.23	0.48	0.13	0.37
35	Muurolol	1651	0.16	0.21	0.19	0.25
36	Bisabolol	1660	0.19	0.23	0.23	0.40
Total			95.34	95.61	96.13	89.42

[a] Kovat's index.

3. Results and discussion

The yellow color of the essential oil from *T. vulgaris* Lamiaceae leaves were obtained in yields of 0.83–1.39% (w/w). The highest oil yield obtained was from S_1 (June-6-2010 sample) with 1.39% (w/w). The lowest amount of leaf oil was in July-16-2010 with 0.83% (w/w). This behavior could be dependent on the beginning of fruiting stage. Fig.1 shows the oil yielding results at different stages of the plant growth. The total amount of the oil depends on the stages of the plant growth. There was an inverse linear relationship between life time and total amount of the oil (Fig.1).

Essential oil components were isolated by hydrodistillation of *T. vulgaris* Lamiaceae leaves, including 36 components (Table 1). The major components were thymol (38.23–63.01%), o-cymene (5.56–15.47%),γ-terpinene (4.43–7.17%), borneol

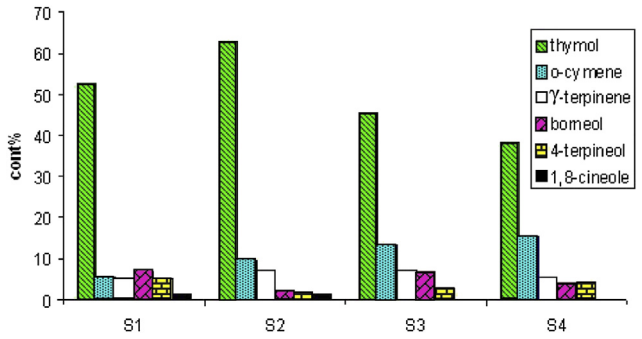

Fig. 2 – A comparison between the main components of *Thymus vulgaris* Lamiaceae leaves oil at different stages of plant growth.

(1.72–6.65%), 4-terpineol (1.24–5.16%), and 1,8-cineole (0.09–1.54%).

Comparing the oil composition of these stages of the plant growth showed some differences (Fig.2). The first major

Table 2 – Comparison of the results of two main components of *Thymus* species essential oil from different countries and present work.

Species	Compound% in the oil	Ref.
Thymus vulgaris from North Khorasan Province of Iran	Thymol (49.85), carvacrol (–)	The average in present work
T. numidicus from Algeria	Thymol (68.2), carvacrol (16.9)	Kabouche et al., 2005
T. fontanesii from Algeria	Thymol (67.8), carvacrol (1.7)	
T. revolutus from Turkey	Thymol (4.6), carvacrol (43.13)	Karaman et al., 2001
T. pallescens from Sidi Aissa	Thymol (1.7), carvacrol (50.9)	Hazzit et al., 2009
T. pallescens from Boussaada	Thymol (1.7), carvacrol (46.9)	
T. pallescens from Oued Rhiou	Thymol (49.3), carvacrol (9.0)	
T. pallescens from Kadiria	Thymol (0.6), carvacrol (44.4)	
T. pallescens from El-Asnam	Thymol (<0.1%), carvacrol (57.7)	
T. algeriensis from Chrea National Park at 800 m altitude	Thymol (29.5), carvacrol (3.3)	
T. algeriensis from Chrea National Park at 1500 m altitude	Thymol (<0.1%), carvacrol (–)	
T. algeriensis from El-Asnam	Thymol (0.2), carvacrol (1.0)	
T. dreatensis from Takoucht	Thymol (20.2), carvacrol (1.1)	
T. maroccanus from South-West of Morocco	Thymol (1.87), carvacrol (89.15)	Saad et al., 2010
T. broussonetii from High Atlas of Morocco	Thymol (39.64), carvacrol (21.31)	
T. carmanicus from Kerman–Iran	Thymol (40.8), carvacrol (24.8)	Nejad Ebrahimi et al., 2008

Table 3 – The seasonal MIC result of T. Lamiaceae against Gram positive and Gram negative bacteria.

Sample	Anti-bacterial (MIC values in μg ml^{-1})										
	Yersinia enterocolitica(E)[a]	*Staphylococcus epidermidis*(P)[b]	*Streptococcus pyogenes*(E)	*Staphylococcus aureus*(P)	*Klebsiella pneumoniae*(E)	*Enterococcus faecalis*(P)	*Escherichia coli*(E)	*Pseudomonas aeruginosa*(E,P)	*Listeria monocytogenes*(E)	*Proteus vulgaris* (E)
S_1	5×10^2	5×10^3	5×10	5×10	5×10^3	5×10	5×10^3	–	5×10	5×10^3
S_2	5×10^3	5×10^3	5×10^3	5×10^3	5×10^3	5×10^2	5×10^2	–	5×10	5×10^3
S_3	5×10^3	5×10^3	5×10^3	5×10^3	5×10^3	5×10^3	5×10^3	5×10^5	5×10^2	5×10^4
S_4	5×10^3	5×10^3	5×10^3	5×10^3	5×10^3	5×10^3	5×10^3	–	5×10^2	5×10^3

The bold values show the lowest concentration of each essential oil with antibacterial effect.

a Entestinal.

b Parchment.

compound that obtained from all of the studied samples was thymol that is confirmed in literature (Nezhadali et al., 2010a,b,c). The highest and lowest percentages of thymol were found in S_2 (63.01%) and S_4 (38.23%), respectively. The minor compound included in all samples was 1,8-cineole. Comparing the essential oils' profile of the leaves, among different stages of plant growth showed a correlation between life time and amount of o-cymene. The amount of o-cymene linearly increases with the plant life time.

The oils obtained from *Thymus* species of different countries and regions (Table 2) showed difference in the amount of the two major components (thymol and carvacrol). Thymol was the major component of *Thymus* species gathered from Morocco, Iran and Algeria (Kabouche et al., 2005; Saad et al., 2010; Nejad Ebrahimi et al., 2008). On the other hand its concentration is very low in El–Asnam and Chrea National Park (Hazzit et al., 2009). In most of the studied cases, there was a linear relationship between concentration of thymol and carvacrol. If thymol is in high concentration in the oil carvacrol amount is low and vice versa.

Based on the high amount of thymol, o-cymene and other terpenoids in the oils of *T. vulgaris* Lamiaceae leaves, it could be concluded that the leaves and its essential oil could be used as flavoring agents in food, medicinal and perfume industries.

Table 3 shows the average MIC values of essential oil from *T. vulgaris* Lamiaceae leaves against the selected bacteria. The MBC results showed that even the most dilute antibacterial sample does not have bacteriocidal effect but bacteriostatic. According to the essential oil definition (Bakkal et al., 2008), the inability of bacteriostatic effect is due to evaporation and lose of essential oil original quality. Therefore, the chemical definition of essential oil could be applied. It is also concluded that the disk diffusion method is a suitable way to assess the antimicrobial effects of the essential oils. The results (Fig. 1 and Table 3) showed that not only the maximum amount of oil yield is for S_1 but also the MIC for most of the studied bacteria is due to S_1 as well.

The essential oils have been showed a great antibacterial effect against *E. faecalis*, *S. aureus* and *S. pyogenes*. These bacteria are Gram positive Cocci. *S. pyogenes* and *S. aureus* are important cause of skin infections. This bacterium has resistance to many antibiotic drugs (Akgul and Kaya, 2004).

As it is shown in Table 4 MIC ($\mu g\ ml^{-1}$) for different *Thymus* species were not the same. MIC of *T. vulgaris* oil is 9.25 for *Escherchia coli* ATCC 25922 but this factor is 5×10^3 for *Klebsiella pneumoniae* PTCC 1053 and *Staphylococcus epidermidis* PTCC 1435 bacteria. The MIC range for 29 studied bacteria is between 9.25 and 1.25×10^4. It seems the poorest antibacterial effect is for *Thymus daenensis* essential oil.

Table 4 – The MIC result of some *Thymus* species against some bacteria.

Bacteria	MIC(μg.ml−1)				Ref.
	Thymus vulgaris	*Thymus numidicus*	*Thymus fontanesii*	*Thymus daenensis*	
Staphylococcus aureus ATCC 25922	1.85×10	NT	NT	NT	Tohidpour et al., 2010
Staphylococcus aureus PTCC 1113	5×10	NT	NT	NT	Present work
Staphylococcus aureus ATCC 25923	1.33×10^3	3.2×10	3.2×10	1.25×10^4	Imelouane et al., 2009·Mojab et al., 2008·Kabouche et al., 2005
Staphylococcus aureus ATCC 29737	NT	NT	NT	1.25×10^4	Mojab et al., 2008
Staphylococcus aureus ATCC 6538	NT	NT	NT	6.25×10^3	
Escherchia coli ATCC 25922	9.25	NT	NT	–	Tohidpour et al., 2010
Escherchia coli ATCC 8739	NT	NT	NT	–	Mojab et al., 2008
Escherchia coli ATCC 35218	NT	NT	NT	–	
Escherchia coli PTCC 1330	5×10^2	NT	NT	NT	Present work
Klebsiella pneumoniae ATCC 13883	5.55×10	NT	NT	NT	Tohidpour et al., 2010
Klebsiella pneumoniae ATCC 700603	NT	NT	NT	–	Mojab et al., 2008
Klebsiella pneumoniae PTCC 1053	5×10^3	NT	NT	NT	present work
Klebsiella pneumoniae	NT	1.6×10^{-2}	1.6×10^{-2}	NT	Kabouche et al., 2005
Bacillus cereus ATCC 9634	37	NT	NT	NT	Tohidpour et al., 2010
Bacillus subtilis	NT	3.2×10	3.2×10	NT	Kabouche et al., 2005
Enterobacter aerogenes	NT	1.6×10^{-2}	1.6×10^{-2}	NT	
Escherichia coli ATCC 25922	NT	1.6×10^{-2}	1.6×10^{-2}	NT	
Proteus mirabilis	NT	3.2×10^{-2}	3.2×10^{-2}	NT	
Pseudomonas aeruginosa ATCC 27853	NT	6.4×10	6.4×10	–	Mojab et al., 2008·Kabouche et al., 2005
Salmonella typhimurium	NT	1.6×10^{-2}	1.6×10^{-2}	–	
Serratia marcescens	NT	1.6×10^{-2}	1.6×10^{-2}	NT	Kabouche et al., 2005
Enterococcus faecalis PTCC 1394	5×10	NT	NT	NT	Present work
Enterococcus faecalis ATCC 29212	NT	NT	NT	1×10^5	Mojab et al., 2008
Micrococcus luteus ATCC 9341	NT	NT	NT	1.25×10^4	
Streptococcus pyogenes ATCC 8668	NT	NT	NT	3.12×10^3	
Streptococcus pyogenes PTCC 1447	5×10	NT	NT	NT	Present work
Staphylococcus epidermidis PTCC 1435	5×10^3	NT	NT	NT	
Staphylococcus epidermidis ATCC 12228	NT	NT	NT	1.33	Imelouane et al., 2009
Panteoa sp.	NT	NT	NT	6.6×10^{-1}	Imelouane et al., 2009

NT.not tested–no activity against bacteria.

According to the previous results, the present studied essential oil has better effects against Gram positive bacteria than Gram negative ones except *S. epidermidis*.

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